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R2D2 Leads the Silencing Trigger to mRNA's Death Star

During RNA interference (RNAi), Dicer generates short interfering RNAs (siRNAs), which then guide target mRNA cleavage by the RISC complex. Now, Liu et al. identify R2D2, a Dicer-associated protein that is important for siRNA incorporation into RISC, thus linking the initiation and execution phases of RNAi.

For many years, biologists understood very little about how antibodies were specified and produced, but were nonetheless undeterred from immunizing animals and making excellent use of the resulting antisera. Immunologists have since narrowed this gap between understanding and utility, but now an analogous situation has arisen in a completely different field. Biologists of nearly every stripe are using RNAi to inactivate and characterize specific genes, in spite of our primitive understanding of the underlying mechanisms. Liu et al. (2003) have now laid the groundwork for clarifying a fundamental mechanistic issue in RNAi: how the double-stranded RNA (dsRNA) silencing trigger makes the transition from nuclease substrate to nuclease specificity factor.

RNAi is a member of a newly discovered set of gene control pathways that all share a common feature: they sequence-specifically silence the expression of genes in response to dsRNA (see Denli and Hannon [2003] for a recent review). The RNAi pathway downregulates gene expression at the level of mRNA stability, and the sequence specificity of mRNA destruction arises from the sequence of the dsRNA silencing trigger itself. A wide variety of eukaryotic organisms are capable of mounting an RNAi response, and often appear to use this pathway to defend against viruses and transposable elements. In the initiation step of RNAi, Dicer, a member of the ribonuclease III family, processes the dsRNA trigger into 21–23 nucleotide duplex siRNAs (Bernstein et al., 2001). Subsequently, the siRNAs are incorporated into the RNA-induced silencing complex (RISC), where they guide the site-specific cleavage of complementary mRNA (Hammond et al., 2000; Zamore et al., 2000). Very little is known about the physical and functional relationship between the initiation and effector phases of RNAi.

One of the best-developed biochemical systems for RNAi employs extracts from cultured *Drosophila* S2 cells (Hammond et al., 2000). Liu et al. (2003) used this system to begin to characterize the dsRNA processing machinery and its links with the RISC complex. After fractionating dsRNA processing activity through multiple chromatographic steps, the authors identified the components of the purified active fractions, and immediately came up with their first surprise: siRNA production was associated most strongly with one of the two *Drosophila* Dicer isoforms (Dicer-2) but not the other (Dicer-1). This conclusion was subsequently confirmed by Dicer-isoform-specific knockdowns in S2 cells, though a significant role for Dicer-1 in siRNA production in other cell types cannot be excluded from these results alone.

Liu et al. (2003) then turned their attention to a distinct 36 kDa protein that cofractionated with dsRNA processing activity. Sequence analysis revealed a previously uncharacterized protein with tandem dsRNA binding domains; the authors dubbed this protein R2D2. Antibodies raised against R2D2 codepleted Dicer-2 but not Dicer-1 from S2 extracts, confirming that R2D2 and Dicer-2 form a stable complex. Significantly, R2D2 displays ~33% sequence similarity to the Dicer-associated *C. elegans* protein RDE-4 (Tabara et al., 2002), which was originally identified genetically as an RNAi factor that is important for the initiation but not the maintenance of RNAi (Tabara et al., 1999; Grishok et al., 2000). Homozygous *r2d2* mutant embryos (generated by P element mobilization) were refractory to RNAi in response to injected dsRNA, confirming the role of R2D2 in RNAi in vivo.

What is the role of R2D2 in the initiation of RNAi? Intriguingly, purified recombinant Dicer-2 catalyzed siRNA production with equal efficiency in the presence or absence of R2D2. In contrast, gel-shift and UV cross-linking assays revealed that the Dicer-2/R2D2 complex (but not Dicer-2 alone) associated stably with siRNAs; furthermore, siRNA binding by the Dicer-2/R2D2 complex was nearly abolished by mutations in the dsRNA binding domains of R2D2. The apparent role of R2D2 in siRNA retention *after* processing provided the first indication that R2D2 controls siRNA fate rather than production, prompting the idea that R2D2 participates in channeling siRNAs into the RISC complex.

In previous work with S2 cell extracts, the dsRNA trigger was added to the live cells before extract preparation (Hammond et al. 2000). However, Liu et al. (2003) found that coupled siRNA production and target mRNA cleavage can occur entirely in vitro in S2 cell extracts, and furthermore showed that the siRNA production machinery could be crudely but effectively separated from RISC complexes by polyethylene glycol (PEG) precipitation. These observations set the stage for the critical experiments to test the requirement for the Dicer-2/R2D2 complex in the effector phase of RNAi. Addition of Dicer-2 alone to the RISC-containing PEG supernatant stimulated modest RISC activity in response to a long dsRNA trigger, but maximal stimulation was only observed upon addition of the Dicer-2/R2D2 complex. Mutation of R2D2's dsRNA binding domains abolished the

stimulation of RISC activity. Importantly, similar effects were observed when pre-cleaved siRNAs were used to trigger RISC, indicating that RISC does not efficiently utilize siRNAs in the absence of R2D2. Finally, the authors drove this last point home by showing that the known RISC component Argonaute2 (Hammond et al., 2001) is not efficiently coselected with biotinylated siRNAs unless functional Dicer-2/R2D2 complex is present. The clear implication is that R2D2 helps to mediate the transition between the initiation and execution phases of RNAi.

Naturally, the identification of R2D2 (and its role in bridging the two main stages of RNAi) raises a new set of questions. Exactly how does R2D2 funnel siRNAs from Dicer-2 to RISC? Is this an active, energy-requiring process? Does R2D2 accompany siRNAs into RISC, or does it simply hand them off? siRNAs with non-native structures (e.g., with 5′-hydroxyl termini) have been shown to be excluded from RISC, implying that certain structural features such as 5′-phosphates may license bona fide siRNAs for RNAi (Nykänen et al., 2001). Now that R2D2 has been shown to be intimately involved in loading siRNAs into RISC, does R2D2 help to discriminate against siRNA impostors? The results of Liu et al. (2003), combined with the experimental tools that now exist (e.g., anti-R2D2 antibodies, recombinant R2D2 protein, and *r2d2* mutant flies), foreshadow rapid progress in answering these and other fundamental mechanistic questions about the RNAi pathway.

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